New Isolates of Pneumonia Virus of Mice (PVM) from Japanese Rat Colonies and their Characterization

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Abstract: Two virus strains were isolated from the lungs of athymic rats and mice used as sentinel animals in 2 colonies of laboratory rats in Japan in which antibodies to the pneumonia virus of mice (PVM) had been detected. The new isolates were identified as PVM by the following characteristics: RNA virus, susceptibility to ether treatment, long filamentous viral structure in the cytoplasm of infected cells, and hemagglutinating activity in various erythrocytes, including those of mice and rats. In addition, cross neutralization with the prototype of PVM (No.15 strain) was observed. This is the first report of the isolation of PVM from laboratory animals in Japan.

Key words: mouse, PVM, rat, RNA, virus

Introduction

Horsfall *et al.* [8] isolated pneumonia virus of mouse (PVM) from the lungs of healthy mice during etiological studies of human pneumonia in 1939. Natural PVM infection is widespread in such laboratory rodents as mice, cotton rats, gerbils, and guinea pigs [8, 10, 14, 17, 23], and serological evidence has also suggested the infection in laboratory rabbits and man [5, 15, 18]. The dissemination of PVM from mouse colonies in the United States, Canada and Europe has also been reported [13, 14, 16]. PVM was originally considered to cause latent or asymptomatic infection in mice [9], but subsequently spontaneous clinical disease caused by the

virus has been described in immunodeficient mice. It was recently shown that athymic mice spontaneously or experimentally infected with the virus died of dyspnea [2, 20, 24]. Furthermore, mice inoculated with tissue culture-adapted virus developed clinical disease characterized by weight loss, labored respiration, and death due to pneumonia [2]. This finding suggested that this virus could induce respiratory disease under certain conditions, thus interfering with long-term animal experiments.

In Japan, the immunofluorescent antibody (IFA) test has shown that PVM is widespread in rat colonies [15] but, in general, PVM has not been examined by standard serological monitoring in breeding colonies of SPF

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mice and rats, except in the colonies of major breeders, although two research groups have reported the detection of hemagglutination-inhibiting (HAI) antibody in mouse and rat colonies [11, 12]. In this study, PVM was isolated from rat colonies. The basic characterization of the isolates is described.

Materials and Methods

Sentinel animals in rat colonies: For virus isolation, euthymic Wistar (♂) and athymic F-344 rats (rnu/rnu, ♂) purchased from CLEA Japan (Jcl; Tokyo) were placed in PVM-positive rat rooms as sentinels at 4 weeks of age (Colony N). Similarly, seronegative euthymic ICR mice (♂), at 4 weeks of age, were placed in a commercial rat breeding colony (Colony C). Four-week-old ICR mice (♂, Charles River Japan, Yokohama) were employed for mouse antibody production (MAP) test. All these mice and rats were free of evidence of PVM infection.

Colony N, which was maintained under conventional conditions in a research facility, has been free of evidence of HVJ and mycoplasma pulmonis infections. A euthymic (Et) rat sentinel group was maintained for antibody monitoring, and an athymic (At) rat group was maintained for virus isolation. The animals were placed near an exhaust air duct in rooms where PVM infection had been found to be prevalent serologically among the rat population. The Et rat sentinel group was placed in the rat room 5 days earlier than the At rat sentinel group. The sera were collected from retroorbital plexus of three rats in the Et group which were anesthetized with diethyl ether at 3-day intervals. One rat in the At group in the N colony was sacrificed each day and the lungs were collected for 7 days, beginning from the day the rats in the Et group seroconverted.

In colony C, which had been maintained as a commercial SPF rat breeding colony, only the At sentinel mouse group was introduced for direct virus isolation. Three At mice were sacrificed and their lungs were collected on days 4, 7 and 14 after settlement.

The animal experimentation procedures followed the Guidelines of the Animal Care and Use Committee of Nagasaki University.

Virus, medium, and cells: The PVM prototype (No.15 strain), obtained from The American Type Culture Col-

lection (Rockville, Maryland, U.S.A.), was propagated in BHK-21/clone 13 cells and used after 8 passages. Murine cytomegalovirus (MCMV), obtained from Dr. Y. Minamishima of Miyazaki Medical College, Miyazaki, was propagated in the same cells. The growth and maintenance media used were: minimum essential medium (MEM; Gibco Co., Grand Island, New York, U.S.A.) containing 5% heat-inactivated fetal calf serum (FCS; Hyclone Lab., Logan, Utah, U.S.A.) and antibiotics (MEM-5).

Four cell lines were employed: BHK-21/clone 13, BSC-1 (obtained from Japanese Cancer Resources Bank, Tokyo), B95a and MA-104 cells (obtained from Drs. F. Kobune and A. Hasegawa of the National Institute of Health of Japan).

Preparation of lung homogenate: Lungs were separated, suspended in MEM-5 at a final concentration of 10% (w/v), and homogenized in an ice-bath for 1 min at 4,000 rpm in a biological safety cabinet (Class II). Aliquots of homogenate were centrifuged at 2,000 rpm at 4 °C for 10 min. The supernatants were used for the MAP test and for virus isolation.

Virus isolation in cell culture: Two ml aliquots of twofold diluted 10% lung homogenate were inoculated into the monolayers of the four cell lines in a 30 ml plastic bottle. After adsorption at 37 °C for 2 hr, the cells were fed with 6 ml of MEM-5 and cultured for 1 week in a CO₂ incubator at 37 °C. The culture media were collected 7 days post infection (dpi) and stored at -80 °C. Viral antigen was determined by the IFA test, as previously described for LCM [21] and PVM [15]. Anti-PVM (strain No. 15) rat serum was employed as the first serum.

Mouse antibody production (MAP) test: Samples (0.4 ml) were inoculated intraperitoneally (i.p) in two ICR mice. Three weeks after inoculation, the mice were anesthetized with diethyl ether and bled by heart puncture. The MAP antibody titer in the sera was then measured by the IFA test.

Virus titration and detection of viral antigen: BHK-21 cell monolayers in 96-well flat plates (Costar Co., Cambridge, Massachusetts, U.S.A.) were inoculated with test samples diluted 1:10 with MEM-5. After adsorption for 2 hr at 37 °C, the inoculum was aspirated and the cells were re-fed with 100 µl of MEM-5. At 7 dpi, the media were removed and the cells were air-dried for 1 hr at room temperature in a safety cabinet.

The plates were fixed for 1 hr with 200 μ l of 10% neutral formalin, and examined by the Avidin-biotin-peroxidase complex method (ABC; Vector Lab., Burlingame, California, U.S.A.). Anti-PVM (strain No.15) rat serum with 10 HAI units was used as the first serum. The 50% tissue culture infectious dose (TCID₅₀) was calculated.

Hemagglutination (HA) activity: ICR mouse, guinea pig, Wistar rat, Syrian golden hamster, Japanese white rabbit, New Hampshire chicken, and human-O type erythrocytes were used for the HA test. Fifty micro-liters of 0.5% erythrocyte suspension was mixed with an equal volume of serial twofold dilutions of virus suspension. Dulbecco's PBS (+), containing 0.1% bovine serum albumin, 0.01% gelatin and 0.02% NaN₃ was used as the standard diluent. After reaction for 1 hr at 22–25 °C, HA titers were recorded as a reciprocal of the highest dilution of virus that showed a clear agglutinating pattern.

Identification of isolated virus: New isolates were thoroughly examined to determine the type of viral nucleic acid, sensitivity to ether treatment, hemadsorption (HAD) activity and serological cross-reactions using viral neutralization (VN) and HAI tests. Viral nucleic acid was determined by staining with acridine orange, as described by Gomatos *et al.* [6], and also by treatment with 5-iodo-2'-deoxyuridine (IUdR; Wako Chemicals Co., Tokyo), as described by Tamm *et al.* [22]. The ether sensitivity test was employed to confirmed whether the isolates were those of an envelope virus [3]. The HAI test was performed according to a previously described procedure [15]. For HAD, the infected cells were examined 1 week post-infection. The cells were gently rinsed 2 times with PBS(–). After the addition of 0.5% erythrocyte suspension in MEM, the cells were incubated for 1 hr at 22–25 °C. Unadsorbed erythrocytes were rinsed out 2 times with PBS(–). The VN antibody test was then performed, as follows: Stock virus, of approximately 200 TCID₅₀, was mixed with an equal volume of serial twofold dilution of mouse serum (ranging from 1:10 to 1:640) which had been heat-inactivated at 56 °C for 30 min. The virus-serum mixtures were incubated at 37 °C for 1 hr and subsequently at 4 °C for 18 hr. Fifty μ l aliquots were used to inoculate 4 wells for each dilution. VN antibody titers were expressed as a reciprocal of the highest serum dilution at which virus infection was in-

hibited in at least 2 of the 4 wells.

Enzyme-linked immunosorbent assay (ELISA): Detection of antibody to HVJ, mouse hepatitis virus (MHV) and mycoplasma pulmonis was done with a commercial ELISA kit (MONILISA®; Wakamoto Pharmaceutical Co., Tokyo).

Density gradient centrifugation: Supernatants obtained from the infected BHK-21 cells were centrifuged at 5,000 rpm for 30 min and then ultracentrifuged for 2 hr at 100,000 $\times$ g. The pellets were resuspended in 1 ml of PBS (–), layered on the top of a 15% to 60% (w/v) linear sucrose gradient in 0.2 M Tris-HCl buffer (pH 7.4), and ultracentrifuged for 16 hr at 100,000 $\times$ g. Two hundred- μ l fractions obtained from the upper portion were examined for HA activity and virus infectivity. Density was determined by weighing 10 μ l of each sample.

Electron microscopy: Samples purified by sucrose density gradient centrifugation were dropped onto carbon-coated collodion membrane grids, dried for 10 min at room temperature, stained with 3% uranyl acetate, and examined under a transmission electron microscope (Type JEM-1200EX; JEOL Co., Tokyo).

Results

Isolation of PVM: Two breeding colonies of rats (codes N and C in Table 1) which had been confirmed to be serologically positive for PVM were selected for the isolation experiment. Taking into consideration our previous experience in isolating HVJ from mice, we initially introduced two groups of sentinels, Et and At rats, into the rat colony N. The Et sentinel rat group was investigated for the detection of seroconversion and the At rat group was investigated to determine virus isolation. The cages of the Et group were placed in the room 5 days before those of the At group. As shown in Table 1, seroconversion was detected in the Et group on day 18 after the placement in the room as room-mate sentinels. The virus isolation experiment in the At group was therefore initiated on the 16th day after the placement of the Et group, and continued for another 6 days. At autopsy, consolidation in the lung was observed in all athymic rats in the At group. MAP antibody titers at levels of 1:2,560–10,240 were usually recognized. Based on these results in colony N, sentinel Et mice were intentionally introduced into the SPF

Table 1. Antibody assay and virus isolation in sentinel rat and mouse groups

Rat colony	dprm ^{a)}	Type of sentinel	Reciprocal IFA titer of sentinels	MAP ^{b)}	Virus isolation
Code N	16	Et ^{c)} rat	<10	+	+
		At ^{d)}	<10	+	+
	17	Et	<10	+	+
		At	<10	+	+
	18	Et	10	+	—
		At	<10	+	+
	19	Et	160	+	—
		At	<10	+	+
	20	Et	160	—	—
		At	<10	+	+
	21	Et	160	—	—
		At	<10	+	+
	22	Et	160	—	—
		At	<10	+	+
Code C	4	Et mouse	<10	NT ^{e)}	—
	4	Et	<10	NT	—
	4	Et	<10	NT	—
	7	Et	<10	NT	—
	7	Et	<10	NT	—
	7	Et	<10	NT	—
	14	Et	20	NT	—
	14	Et	10	NT	+

a) dprm: days post-placement as room-mate. b) MAP: mouse antibody production test. Titer higher than 10 by IFA antibody was taken as MAP; positive.

c) Et: euthymic rats (Wistar rat). d) At: athymic rats (F-344 rat). e) NT: not tested.

rat breeding colony (C) to isolate the virus directly.

The four cell cultures: BHK-21, BSC-1, B95a and MA-104 cells were inoculated with lung homogenates of the At and Et rats and the Et mice from both N and C colonies, and these cells were examined for virus antigen by the IFA test. PVM-specific antigen was detected only in the BHK-21 cells, so that subsequent experiments were conducted only in these cells (Fig. 1). Virus antigen was demonstrated in the cells inoculated with the 16, 17 dpi homogenates in the Et rat group, but the antigen was observed for 7 days in all cells inoculated with the 16 to 22 dpi lung homogenates of the At rat group.

Ten isolates altogether, 9 from colony N and 1 from colony C, were obtained and 2 strains were selected, one as representative of the N colony (strain 31N) and one as representative of the C colony (strain 14-3). The two representative strains were passaged in BHK-

21 cells by a limiting dilution method. Both strains induced moderate cytopathic effects (CPE), showing cell rounding, whereas the prototype strain, No. 15, did not exhibit typical CPE (Fig. 2). No viral plaques were formed in the cells by any of these 3 strains.

Biological and physicochemical characterization of the 2 new isolates: The possibility of viral contamination of the culture fluid by other murine pathogens, such as HVJ, mouse hepatitis virus, and mycoplasma pulmonis, was examined. Mice were inoculated i.n. and i.p. with culture fluids containing $10^{5.0}$ TCID₅₀ of the 2 strains, and their sera were examined by ELISA 3 weeks after inoculation. No seroconversion against any of these murine pathogens was found (data not shown).

HA activity in erythrocytes of various species was also examined. Both isolates demonstrated clear agglutination in mouse, rat, hamster and rabbit erythrocytes, but not in guinea pig, chicken, or human-O type

Fig. 1. IFA test for PVM-specific antigen. BHK-21 cells inoculated with the lung homogenates were stained with anti-PVM (No. 15) rat serum at 7 dpi.

erythrocytes, the results being similar to those seen with the prototype No. 15 strain (Table 2). The HA titers were different in the prototype and isolated strains, in particular, the No. 15 strain showed a low HA titer in rabbit erythrocytes. BHK-21 cells infected with the 2 isolates were found to be HAD-positive to the mouse erythrocytes, similarly to the prototype (Fig. 3).

With the acridine orange method, the cytoplasm of the virus-infected cells was stained orange at 6 dpi. The addition of IUdR to the culture media did not affect the virus yield. The isolates were therefore assumed to be a RNA virus. A marked reduction in viral infectivity was observed after treatment with diethyl ether for 18 hrs at 4 °C, indicating ether-sensitivity (envelope virus), as shown in Table 3.

The buoyant density in sucrose of the isolates was compared with that of the prototype strain, No. 15. As shown in Fig. 4, most infectivity was found at a density of approximately 1.18 g/cm³, whereas hemagglutination activity peak was found at a density of 1.15 g/cm³. These patterns were the same as those of the prototype No. 15. The presence of 2 peaks for HA and the infectivity of the virus suggested that this virus could be labile, resulting in an the easy release of HA activity, as previously shown by Harter *et al.* [7]. The antigenic relationship between the isolates 31N and 14-3 and the prototype, No. 15 was examined by conducting HAI

and VN tests. The three strains cross-reacted with each other, although there was a 4-fold difference in HAI titers between the isolates and the prototype, No. 15 (Table 4).

Electron microscopic findings: The peak fraction of virus infectivity obtained by sucrose density gradient centrifugation was examined by electron microscopy, with negative staining. As shown in Fig. 5a, b, the two new isolates and the prototype retained mainly a filamentous structure, and rarely a spherical or pleomorphic structure. A few spherical forms had a diameter varying from 100 nm to 200 nm. Filamentous forms had a constant diameter of 100–120 nm and a length of 1–3 μ m. The HA peak fraction (density 1.15), which probably represents hemagglutinin, contained structures with a diameter of 10–100 nm (Fig. 5c).

Discussion

Despite serological evidence suggesting contamination of rodent colonies by PVM in many countries [13, 14] including Japan [11, 12], there has, thus far, been no report of the isolation of PVM in Japan. Our serological survey, carried out in a previous study also indicated PVM prevalence in rat colonies in Japan regardless of conventional or barrier conditions [15]. In this study, we isolated new strains of PVM by intro-

Fig. 2. CPE with 31N and 14-3 strains in BHK-21 cells at 5 dpi (high multiplicity of infection). a: uninfected BHK-21 cells ($\times 120$), b: BHK-21 cells infected with 31N ($\times 300$), c: BHK-21 cells infected with 14-3 ($\times 300$).

Table 2. Hemagglutinating activity of the 2 new isolates

Erythrocytes	Prototype	New isolates	
	No. 15	31N	14-3
Mouse	256 ^{a)}	32	256
Rat	32	32	128
Hamster	64	32	128
Rabbit	8	32	256
Guinea pig	—	—	—
Chicken	—	—	—
Human-O	—	—	—

^{a)} HA titers are expressed as the reciprocal of the highest dilution of virus that showed a definite agglutination pattern.

Table 3. Sensitivity to IUdR and ether treatment

Treatment	Virus	Strain	Virus titer ^{a)}	
			Untreated	Treated
IUdR ^{b)}	PVM	No.15	5.7	5.0
		31N	4.7	5.0
		14-3	4.5	4.3
	MCMV ^{d)}	Smith	4.0	0.7
Ether ^{c)}	PVM	No.15	5.7	<0.25
		31N	5.0	<0.25
		14-3	4.5	<0.25

^{a)} Virus titers are expressed as log₁₀ TCID₅₀ per 50 μ l.

^{b)} IUdR treatment was performed as follows: Ten-fold dilutions were inoculated in both IUdR-untreated and treated cells; viral infectivity was then assayed.

^{c)} Viral suspensions were mixed with diethyl-ether or saline and stirred for 18 hr at 4 °C. After treatment, the residual ether gas was removed by bubbling with N₂ gas; viral infectivity was then assayed.

^{d)} Murine cytomegalovirus.

Fig. 3. HAD with 31N strain in BHK-21 cells at 7 dpi (low MOI) ($\times 144$).

Fig. 4. Sucrose gradient centrifugation study. The longitudinal axis indicates HA activity (○), virus titer (●) and density (△). The HA titer is expressed as the highest positive dilution of each fraction.

Table 4. Serological cross reactivity

Antigen (strain)	Test	Mouse antisera		
		vs		
		No. 15	31N	14-3
No. 15	HAI	320 ^{a)}	160	80
	VN	80	160	20
31N	HAI	80	640	320
	VN	20	160	80
14-3	HAI	80	640	320
	VN	20	40	160

^{a)} HAI and VN titers are expressed as the reciprocal of the highest serum dilution.

Fig. 5. Electron microscopical observation with negative staining method. (a) A filamentous viral particle was observed in the fraction of No. 15 strain which high-titered viral infectivity was demonstrated. (b) Two filamentous viral particles and spherical particles in the peak fraction of the 14-3 strain. (c) Viral HA protein (probably envelope protein) observed in the peak of HA titer of 14-3 strain. Bar=500 nm.

ducing sentinels as room mates in rat breeding colonies. Of the sentinels from which PVM was isolated, F-344 nude rats (rnu/rnu) had pulmonary lesions in the lungs (data are not shown), suggesting that PVM exhibits pathogenicity in naturally infected athymic rats.

In regard to virus adaptation to cells, the No. 15 strain has been reported to replicate in Vero cells and to produce plaques in BSC-1 cells [3, 22]. The two new isolates, however, did not produce plaques, although moderate levels of CPE and PVM antigen were exhibited in BHK-21 cells. BHK-21 cells are considered to be suitable for PVM isolation from tissue materials that may contain low virus titers, but viral cytopathogenicity may be low in these cells.

The biological and physicochemical characteristics of the two isolated strains closely resembled those of the prototype, No. 15, although there was a slight difference in HA activity in rabbit erythrocytes and in cytopathogenicity. Since only 3 strains can be compared at present, the existence of a different serotype or a variant type cannot yet be determined. Nevertheless, the similar characteristics of the 31N and 14-3 strains, which were isolated in geographically different colonies in Japan, may indicate the prevalence of one type of PVM in Japan.

The pathological features of interstitial pneumonia in euthymic and athymic mice were consistent with those reported previously [1, 2]. The finding that athymic mice had harbored virus in the lung and showed severe pathological changes suggests persistent infection in these mice, which animals may thus serve as a possible source of infection to other susceptible rodents. Currently, the use of immunodeficient rats (rnu/rnu) and mice (nu/nu; C.B-17/Icr-scid) is increasing in the field of immunology and cancer research. Since our preliminary studies with the new isolates suggest some pathogenicity in immunodeficient mice and rats, we emphasize the importance of identifying non-apparent infections, such as PVM, as well as mouse hepatitis virus (MHV), in mice.

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